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Perspectives

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André Gratia: A Forerunner in Microbial and Viral Genetics

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THE aim of this brief historical account is to show that the genetics of bacteria and their viruses was beginning to take root in Belgium quite early in the century. The comments concern: (a) a revisited history of bacteriophagy in the early period; (b) the spontaneous origin of mutations, *e.g.*, causing colicin or phage resistance in *Escherichia coli*, and microbial variations in general; (c) the discovery of phage mutations affecting lysogeny; (d) a little-known aspect of the history of bacteriophages in connection with the study of viruses and cell biology; and (e) unknown aspects of lysogeny and colicinogeny described long ago and possibly connected with new findings on imprinting in bacteria.

Microbiology has undeniably played a major role in the development of molecular genetics since the beginning of the century, especially since the discovery of bacteriophages shortly after Mendel's laws were rediscovered. Where would genetic engineering be today without the bacteriophage λ or the plasmid ColE1 from which various bacterial cloning vectors derive? Bacteriophages and colicinogenic factors first appeared in work on phages, particularly temperate ones, and on colicins, whose genetic determinants are plasmid borne. Among the pioneers of lysogeny and colicinogeny, Belgian researchers J. Bordet, A. Gratia, P. Fredericq, and R. Thomas stand high beside French scientists F. d'Hérelle, M. Lisbonne, A. Lwoff, F. Jacob, E. and E. Wollman, and their son Elie Wollman. The molecular biology of phages and plasmids has been the subject of many reviews, principally in the English-speaking literature, but little is known about the time before 1950. The fragmentary information from this era is sometimes inaccurate. Therefore, on the occasion of the fiftieth anniversary of the death of André Gratia (1893–1950), I analyze his work among contributions of other French-speaking microbiologists of his time in a book to be published on that early period (*Artisans Belges de la Microbiologie, précurseurs de la Biologie moléculaire*). I have become aware of this information gap as Gratia's son and as a contemporary microbiologist. In this note, I limit my analysis to the surprising contributions to genetics of this imaginative microbiologist. In reality, the observations he records are those of a forerunner, and in several cases later work represents rediscoveries.

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HISTORY OF A CONTROVERSY

When people spoke of microbes in the early 1900s, they were thinking almost exclusively of bacterial (and viral) pathogens affecting humans. Of course, Antonie van Leeuwenhoek's "diertjes" or "animalcules" were present in his flower pots, but when much later Pasteur's pathogenic germs were discovered, no connection was made. When the bacteriophage was discovered, this was a turning point for bacteriology, but phages long remained the concern of medical bacteriologists. The generality of bacterial virology was not noted until much later. This is the context in which Gratia successfully demonstrated the validity of the current view.

In 1920, André Gratia left the Laboratory of Physiology at the Université Libre de Bruxelles, where he was studying blood clotting (staphylocoagulase), and went to work at the Rockefeller Institute for Medical Research in New York. He was attracted by the strong personality of J. Loeb, and he befriended J. Northrop and bacteriologist P. de Kruif. During Gratia's stay in New York, a chance happening led him into the world of bacteriophages. His colleague Peter Olitsky mentioned to him an article on Meningococcus in The Lancet. As Olitsky could not recall the exact reference, Gratia leafed through the 1915 volume and happened upon Twort's (1915) article. Reading it, he became convinced of a link between Twort's "glassy" transformation of Micrococcus colonies by a filtrable agent contained in a "vaccinia lymph" (his words) and the bacteriophage of d'Hérelle (1917), which caused lysis of Shigella cultures. Gratia set to work immediately and showed that the filtrable

agent present in a vaccinia pulp could lyse broth cultures of Staphylococcus. Conversely, he showed that the anti-staphylococcus phage prepared according to d'Hérelle caused naturally opaque Staphylococcus colonies to become glassy. From then on, he viewed these observations as two different manifestations of the same type of agent (just as static electricity and magnetism are two manifestations of the electromagnetic force). Yet it took another 10 years for the plurality of bacteriophages and phenotypic differences in phage activity to be recognized. When, in 1930, Twort wrote, "Gratia showed a beautiful series of experiments in which he used the same filter-passing agent under varying conditions of experiment, and obtained the one or the other manifestation of the phenomenon according as he varied those conditions" (Twort 1930, p. 1070), d'Hérelle still did not accept that bacteriophages are everywhere and that Twort's observations on another bacterium were a case of bacteriophagy.

In the course of the work just described, Gratia made a series of observations whose interest has not caught the attention of most chroniclers. In his book *Arrowsmith*, Sinclair Lewis (1925) thanks de Kruif for having provided information on medical and bacteriological matters and on scientific circles. Gratia, it seems, was one of the models for the composite character Arrowsmith. Abedon (2000) reported that J. J. Bronfenbrenner started phage research in the USA in 1924. Yet, in fact, Gratia appears to have been the first phage research worker in the USA. His work on phages of Staphylococcus and *E. coli* was published in 1921 in the *Journal of Experimental Medicine* and the *Proceedings of the Society for Experimental Biology and Medicine* (Gratia 1921a,b). These medical journals of The Rockefeller Institute were not habitually read by geneticists, as Lederberg (1994) has pointed out, so Gratia's articles were hardly quoted at all.

Thus, Gratia became interested in phages through the circumstance I mentioned above. It was not until after his stint at The Rockefeller Institute that he joined Jules Bordet, who was studying bacteriophages independently at that time at the Institut Pasteur of Brussels. There, although Gratia agreed at first with Bordet's views on phages (as attested by the communication presented at the Glasgow Meeting in 1922), he gradually distanced himself from them. One hypothesis put forward by Twort (1915) was that the agent of "transmissible lysis" is a bacterial enzyme that stimulates its own production. Bordet (1931) favored this hypothesis. According to Twort's other hypothesis, which has since prevailed, a phage is a bacterial virus. Gratia was among those who finally adopted this view. Against the backdrop of controversy over the nature of phenomena involving bacteriophages, Gratia and co-workers developed an intense research activity, summarized here.

First, at the Institut Pasteur du Brabant, Gratia and Bernice Rhodes (Gratia and Rhodes 1923) discovered staphylococcal isophagy, i.e., the lysis of dead staphylococci by living staphylococci. The belief that this phenomenon and bacteriophagy might have a general significance led Gratia to undertake from 1924, notably with Sarah Dath, a systematic search for microbes displaying bacteriolytic activity in nature. In this work, he developed selection techniques that were to be exploited by "antibiotic hunters" (Gratia 1947). Colicin V, described in Gratia's (1925) famous note, was the first among the bacteriocins, a class of antibiotics shown to be related to bacteriophages (see Jacob *et al.* 1953; Fredericq 1958).

A. Gratia at his centrifuge. Photograph by G. Rulmont, courtesy of the author.

In 1932, Gratia was promoted to be head of the Laboratory of Bacteriology of the University of Liège. There he compared phages with plant and insect viruses (Gratia 1939a). He collaborated with specialists of various disciplines, including the Belgian plant pathologist P. Manil, the French insect virologist A. Paillot, and, during World War II, the already famous molecular biologist J. Brachet, to create a general view of virology (see Gratia 1945, 1948). To do this, he introduced some of the most advanced techniques of the time, notably fractionation by centrifugation as early as 1934, using Henriot's famous compressed-air ultracentrifuge (visible in the photograph). As R. Thomas has pointed out to me, R. Jenner's work on *Bacillus megatherium* and plant viruses was largely influenced by the work started

with Gratia. More globally, Gratia's work signaled the emergence of two fields that were to develop so impressively over the 20th century: virology and antibiotics.

THE PROBLEM OF MICROBIAL VARIATIONS AND THE DARWINIAN ORIGIN OF MUTATIONS

Gratia's early work on resistance to phages gave an early glimpse of a fundamental issue in bacterial genetics. In 1923, he used two phages distinct by serology, by heat sensitivity, and by plaque morphology (Gratia 1923). From a culture where these phages formed turbid plaques (*principe faible*), the one forming large plaques and the other small plaques, he isolated Rough and Smooth bacteria. Only Rough bacteria were resistant to the phage isolate forming the large plaques, and only Smooth bacteria were resistant to the small plaque-forming phage. These findings led to two interesting conclusions. First, they showed that distinct phages can have different properties and specificities, reproduced in the progeny of phages isolated from well-separated plaques. Second, since the rough-smooth variation was observed in the absence of any phage, it had to be that the two phage resistances were genetically distinct characters arising independently of the agents used to select the resistant bacteria. Similar findings were described afterward, the best known being that of Burnet (1929) in Salmonella. At the 3rd Congress of Microbiology in New York, 1939, Gratia declared, as Darwin might have done: "Adaptation by passive selection of pre-existing variants is the only fact to be proven beyond any doubt" (Gratia 1939b).

In 1945, P. Fredericq, together with Gratia, started the study of colicins, which were then found to have multiple types, each, like phages, endowed with specific receptors (Fredericq 1946; Gratia and Fredericq 1946). In 1948, he applied the fluctuation test to mutations conferring resistance to various colicins. In 1950, another of Gratia's co-workers, M. Welsch, had analyzed streptomycin resistance in Staphylococcus (Welsch 1950). He interpreted his statistical results on the high level of resistance as the expression of a spontaneous mutation. Yet, as he himself pointed out, the rate of mutation was so low that Luria and Delbrück's fluctuation test was not readily applicable. The decisive evidence came with Lederberg's sib selection experiment in 1952, using replica plating (Lederberg 1989). It showed once and for all that the genesis of streptomycin-resistant mutants in *E. coli* K12 is independent of the presence of the antibiotic in the medium.

Variations such as *rough-smooth* are of great interest to medical bacteriologists and epidemiologists, because the two forms may differ in their pathogenicity. A little-known example is the case of *B. anthracis*, in which Gratia (1924) contradicted the results of Besredka (1921). According to the latter, the skin is the only organ sensitive to anthrax in rabbits and guinea pigs, the other organs being naturally resistant to infection. Gratia noted that *B. anthracis* is subject to variation. He compared two variants, an agglutinating type and a "diffuse" type. He showed that the former, as a rule, caused a fatal septicemia when injected intravenously, whereas the latter seldom produced such a reaction. He obtained evidence that rabbit organs other than skin can be sensitive to the agglutinating variant.

PHAGE MUTATIONS, AN EARLY STEP IN THE GENETICS OF LYSOGENY AND OF VIRUSES

Gratia's abiding interest in variation of both bacteria and their phages led to a key discovery in the analysis of lysogeny. In 1936 he was working with phage lysates of a lysogenic *B. megatherium* strain 899 (the one Lwoff used for prophage induction experiments). When used to infect a sensitive, nonlysogenic homologous strain, the phages in these lysates normally produced turbid plaques. But Gratia observed that phage from each of the turbid plaques he checked could generate a very few clear plaques. He then established that the "clear-plaque" and "turbid-plaque" characters were maintained in the progeny of phages from well-isolated plaques, and he observed that only the turbid-plaque phage was able to lysogenize the sensitive bacterial strain. He was early to recognize this variation as a case of mutation in phage (Gratia 1936a,b). The same year, Burnet and Lush (1936) also described a clear-plaque phage mutant of Staphylococcus, but Gratia went one step further. He was able to isolate from clear-plaque lysates a second-step mutant also capable of lysing the lysogenic strain producing the turbid-plaque phage with formation of clear plaques. The importance of these almost unknown observations did not emerge until 1950, after his death, with the work of A. Lwoff, G. Bertani, later A. D. Kaiser, F. Jacob, Elie Wollman, and, still later, R. Thomas (see Lwoff 1953; Thomas 1993). It is now known that phages produced by lysogenic bacteria are of the *temperate* type and that part of the infected bacteria do not lyse but maintain the phage in a latent form called a prophage (*lysogenic response*). The prophage confers immunity to the phage, so that the plaques appear turbid owing to growth of immune bacteria. The clear-plaque *c* phage is important because its phenotype means it can no longer confer synthesis of *an immunity repressor* to infected bacteria, so that only a *lytic response* to the phage remains possible. In some *c* mutants, one or several additional mutations can lead to resistance to the immunity repressor, so that the resulting mutant phages, called *vir* for virulent (but not to be confused with naturally virulent phages that kill bacteria with a lethal colicin-like protein), become capable of growing in a lysogenic bacterium of the same immunity specificity. Thus, the isolation of first-step *c* mutants and second-step *vir* mutants should have been a

major revelation in 1936, especially since Gratia already noted that each wild-type phage particle was potentially virulent, inferring the existence of a genetic trait independent of the bacterium. He even drew a parallel between bacteriophage mutations and the antigenic variations of the influenza virus.

Another 10 years elapsed before M. Delbrück, S. E. Luria, A. D. Hershey, G. Doermann, and then S. Benzer isolated and exploited mutants of the nontemperate T-even phages in mixed-infection experiments. Even then, the Phage Group of Caltech did not believe in lysogeny and ignored previous contributions of phage workers interested in it. For this group, lysogeny reflected carrier cultures composed of bacteria heterogeneous in their sensitivity to phages. Such a possibility does exist, but does not apply to the so-called temperate phages, which, like λ, do not always multiply after infection, as explained above. However, the Phage Group did provide an important change in microbial genetics with high-resolution methods of genetic mapping, T4 phage *rII*, lysozyme, and bacterial metabolic genes, opening the way to cellular and viral genetics (see Fredericq 1957; Stent 1963).

FROM VIROLOGY TO MOLECULAR GENETICS

Burnet's and Gratia's observations in 1936 that mutations can affect some viral functions without altering essential ones were an indication that viruses possess a genome containing several genes, of which some can be altered. Might molecular genetics have developed earlier from such findings? Although Schlesinger (1936) produced some evidence that phages might contain DNA, no link was established between DNA and the carrier of genetic determinants. Even the famous experiments of Avery *et al.* (1944) on transformation of pneumococci, demonstrating that DNA is the genetic information-carrying molecule, did not receive immediate support (see Lederberg 1994). In Belgium, as late as 1945, Gratia's group observed that nucleases (RNase and DNase) had no effect on four tested bacteriophages of various origins (Jeener *et al.* 1945). Therefore, these scientists remained perplexed as to the existence and significance of nucleic acids in bacteriophages. Not until 1952 could the physical structure of phage particles be studied by electron microscopy, showing that the nucleic acid is protected against nucleases by an outer capsid. It took the molecular labeling experiments of Hershey and Chase (1952) and Sinsheimer *et al.* (1962) to convince geneticists that double-stranded DNA of phage T2 or single-stranded DNA of phage ϕX174 is the germinal substance entering a bacterium upon infection by a phage.

Gratia and colleagues were luckier with the grasserie virus of the silkworm. In parallel to A. Claude, who was studying the Rous sarcoma virus and had discovered that noninfected cells contained nucleoprotein granules distinct from the viruses formed in infected cells, Gratia and Paillot (1939) showed that *Bombyx mori* tissues uninfected by the polyhedrosis virus contained granules antigenically distinct from the viruses abundantly formed in infected tissues. Moreover, unlike the Rous sarcoma virus, which contains RNA like the cytoplasmic nucleoproteins (Claude 1940), the granules of infected and uninfected silkworm tissues could be distinguished by their RNA content from the DNA-containing virus granules present only in infected tissues (Gratia *et al.* 1945).

PHAGES, COLICINS, AND PLASMIDS: ECLIPSE IN PHENOTYPIC EXPRESSION

Colicins form a particular group of antibiotics characterized by their protein nature and by the facts that their synthesis is lethal and that adsorption requires specific receptors in the envelope, some receptors being also used by phages, indicating a common ancestry for these agents. For example, certain protein receptors are common to a colicin and a virulent phage (Fredericq and Gratia 1949); others, like the *fig* product, can adsorb a bacteriocin, virulent phages of distinct origins, and even a temperate phage (J. P. Gratia 1989). Fredericq, who resumed the study of colicins in 1945, established the cytoplasmic nature of several factors responsible for colicin production (Fredericq and Betz-Bareau 1953; see Fredericq 1969; Hardy 1975; Luria and Suit 1989) and was the first to integrate the study of colicins into plasmid genetics. Among the colicinogenic factors investigated was the ColE1 plasmid. This plasmid consists of a small, circular DNA molecule carrying only one *Eco*RI restriction site. Its copy number can be increased to about 1000 per cell by exposing the culture to chloramphenicol. Today it is used extensively in molecular genetics and biotechnology.

Gratia (1932) compared two *E. coli* strains, his colicinogenic "coli V" and the lysogenic Lisbonne and Carrère strain. He established a parallelism at several levels between phages and colicins. However, he also made a fundamental distinction between these two types of agent, since only phages are capable of genetic continuity. After his death, the parallelism between these factors was confirmed in several ways (Bordet and Beumer 1951; Fredericq 1952; Jacob *et al.* 1953; Latarjet and Fredericq 1955). From 1926 to 1932, Gratia observed a peculiar activation/reactivation phenomenon that I find especially tantalizing. In both strains the studied character, production of colicin by coli V or of phage by coli Lisbonne-Carrère, could disappear and then reappear. Decades later, noncomplementing diploidy was discovered in hybrids of genetically marked strains obtained either by artificial fusion in *B. subtilis* (Hotchkiss and Gabor 1980; Guillen *et al.* 1983, 1986) or by "spontaneous *zygogenesis*" in *E. coli* (J. P. Gratia 1994, and

manuscript in preparation). It has been shown in these cases that a prophage present in one parental strain or a plasmid used to transform one parent is subject to repeated inactivation and reactivation during the descent of stable, phenotypically haploid, heterozygotic noncomplementing diploids.

HISTORICAL COMMENT AS AN EPILOGUE

At the time of their first studies on antibiosis, Gratia and Dath (1925) mentioned that a Penicillium strain exerted a highly bacteriolytic activity against anthrax-causing bacteria. Gratia's attention was then diverted to the study of "coli V." Unfortunately, a serious illness prevented him from studying this antibiosis due to Penicillium. Once back in his laboratory in 1929, he found that his strain had died. The substance produced was thus never identified. Was it a β-lactam? We shall never know. Yet what would have happened if he had made the opposite choice, to abandon the study of coli V in favor of Penicillium? Thinking of the homage paid to him by foreign scientists such as Alexander Fleming, Roger Herriott, Salvator Luria, and Wendell Stanley, in letters sent to the Université de Liège in October 1950 on the occasion of his death, one is tempted to say that André Gratia made the right choice.

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